

The MAGE A1-A10 Expression associated with Histopathological Findings of Malignant or Non-Malignant Cells in Peripheral Lung Tumors

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Abstract

Objective: The objective was to evaluate the expression of melanoma antigen (MAGE) A from A1 to 10 (A1-10) and the individual MAGE A family in the peripheral lung tumors and to analyze its association with histopathological findings. **Methods:** A cross-sectional study was conducted on 67 samples of peripheral lung tumor obtained by core biopsies from patients with clinical diagnoses such as lung and mediastinal tumors. The specimens were divided into two, one to perform histopathological diagnosis and the last for mRNA MAGE A examination. A Nested polymerase chain reaction (PCR) was performed using universal primer, MF10/MR10 and MF10/MR12. The collected data were analyzed by appropriate statistical techniques. **Result:** The histopathological finding showed 41 (61.2 %) of specimens as malignant cells and 26 (38.8 %) of specimens as non-malignant cells. MAGE A1-10 was expressed at 47 (70.1 %) and MAGE A1-6 was expressed at 25 (37.3 %) of specimens. In a malignant cell, MAGE A1-10 and MAGE A1-6 were expressed at 33 (80.5 %) and 19 (46.3 %), respectively. In non-malignant cells, MAGE A1-10 and MAGE A1-6 were expressed at 14 (53.9 %) and 6 (23.1 %), respectively. The MAGE A1-10 and MAGE A8 expressions were significantly associated with histopathological findings of malignant or non-malignant cells. The sensitivity, specificity, and diagnostic accuracy of MAGE A1-10 were 80.5 %, 46.2 %, and 67.2 %, respectively; while for MAGE A8 were 41.5 %, 88.5 %, and 59.7 %, respectively. **Conclusion:** The MAGE A1-10 expression was the most commonly detected and associated with the histopathological finding. Moreover, it was more sensitive and specific and had higher diagnostic accuracy than others. Therefore, the MAGE A1-10 assay may improve the accuracy of the diagnosis of malignancy in peripheral lung tumors.

Keywords: Lung cancer- cancer cell- MAGE A1-10- MAGE A1-6- core biopsy

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Introduction

Lung cancer is the second most common malignancy in worldwide. GLOBOCAN 2020 data shows that new cases of lung cancer are 2.206.771 cases (11.4 %) and are the main cause of death due to cancer with a mortality rate of 1.796.144 (18.0 %) (Sung et al., 2020). Lung cancer is significantly newly detected at an advanced stage and affects patient survival (Sugita et al., 2002). This may be because most of the patients diagnosed are at an advanced stage making it difficult to provide appropriate treatment (Cainap et al., 2021). The difficulty encountered in diagnosing lung cancer at an early stage is the location of the tumor which is difficult to reach. Furthermore, the patient does not feel symptoms because new symptoms

appear after the cancer has reached an advanced stage (Sugita et al., 2002; Cainap et al., 2021). Locations on the periphery or center of the chest cavity that are difficult to reach with existing equipment. Therefore, molecular approaches to assist detection of lung cancer or determine clinical outcomes can be developed in certain regions of the lung tumor (Mazzone et al., 2017), either centrally or peripherally in the thoracic cavity.

One of the possible approaches for diagnosing patients with lung tumors in the peripheral areas is to perform a biopsy. A core biopsy can be performed in the thoracic cavity under ultrasound guidance or computed tomography (Marhana et al., 2021). The specimen obtained by core biopsy is quite adequate and can be used to specify the type of histopathological diagnosis of lung

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cancer and also contributes to the selection of appropriate therapy for lung tumor patients (Huang et al., 2021; Zhang et al., 2022; Marhana et al., 2022). In addition, the risk of complication such as pneumothorax or hemoptysis of core biopsies can be reduced (Yao et al., 2012). Therefore, the sample from the core biopsy of lung tumors could be used in the molecular diagnosis based on PCR techniques.

Melanoma-associated antigen (MAGE) is a tumor antigen that was first discovered in melanoma patients (Meek and Marcar, 2012). MAGE A belongs to the class of cancer/testis antigens which is expressed on cancer cells and germ cells, including the testis, fetal ovary, and placenta (Öunap et al., 2018; Li et al., 2021). Based on the location of the gene on the chromosome and gene expression in the tissue, MAGE is classified into two kinds. MAGE I consists of MAGE A, B, and C, while MAGE II consists of MAGE D (Weon and Potts, 2015; Li et al., 2021). The family of MAGE A gene consists of 12 subtypes that are MAGE- A1, MAGE- A2, MAGE- A3, MAGE- A4, MAGE- A5, MAGE- A6, MAGE- A7 (pseudo gene), MAGE- A8, MAGE- A9, MAGE- A10, MAGE- A11, and MAGE- A12 (Brisam et al., 2016; Mastutik et al., 2021).

The MAGE A gene has been reported that it was expressed in several types of cancer, such as laryngeal cancer (Liu et al., 2020), oral cancer (Pereira et al., 2012), salivary gland cancer (Beppu et al., 2017), gastric cancer (Ries et al., 2008), colorectal cancer (Almutairi et al., 2022), liver cancer (Mastutik et al., 2010; Li et al., 2020), and lung cancer (Sugita et al., 2002; Karimi et al., 2012). Furthermore, in lung cancer, MAGE A3 and MAGE A4 were identified in lung cancer patients with histopathological type of non-small cell lung cancer (NSCLC) (Shigematsu et al., 2010), while MAGE A1 and MAGE A3 were identified in the early stage of carcinogenesis (Chen et al., 2017). The expression of several subtypes of MAGE A3 and A4 in NSCLC was associated with tumor progression, poor survival, and poor outcome (Shigematsu et al., 2010; Chen et al., 2017; Yi et al., 2017).

The Previous study identified expression of MAGE A1 to MAGE A6 (MAGE A1-6) together using nested PCR (Park et al., 2002). They were expressed in papillary thyroid microcarcinoma (Lee et al., 2013), head and neck squamous cell carcinoma (Noh et al., 2016), and in lung cancer (Yi et al., 2017). However, MAGE A8 to MAGE A10 were expressed in NSCLC (Sugita et al., 2002; Tsai et al., 2007) and small cell lung cancer (SCLC) (Sugita et al., 2002) that may improve the finding of the malignant cell on the small specimens of the core biopsy. Our previous study has identified MAGE A1-10 expression by nested PCR using universal primers that were MF10/MR10 and MF10/MR12 (Mastutik et al., 2021). Therefore, the identification of several subtypes of MAGE A that consists of MAGE A1 to MAGE A10 (MAGE A1-10) could increase the diagnostic value and serve as a predictor marker of cancer progression. The objective of this study was to evaluate the expression of MAGE A1-10, MAGE A1-6, and the individual of MAGE A gene, including MAGE A1, A2, A3, A4, A5, A6, A8, A9, and A10 in the peripheral lung tumor and analyses the association

between MAGE A1-10, MAGE A1-6, and individual MAGE A with histopathological finding.

Materials and Methods

The samples collection

An observational study with a cross-sectional approach was performed in Dr. Soetomo Hospital, Surabaya, Indonesia. Samples were the core biopsies specimens collected from patients with clinical diagnoses suffering from lung and mediastinal tumors in the Lung Intervention Room, Diagnostic Center Building from August 2017 to August 2018. This study was approved by Ethics Committee of Dr. Soetomo Hospital, Surabaya, Indonesia with ethical clearance number 497/Panke.KKE/VIII/2017. All subjects participating in this study signed informed consent.

The Inclusion criteria were patients aged 18–80 years, patients had at least one measurable tumor or lesion, lung tumor underwent ultrasound-guided core biopsy for collection of tissue specimens, Karnofsky score was expressed at > 70 %, patients had never received systemic therapy, and patients willing to participate in the study (with informed consent). The exclusion criteria were the presence of a primary tumor in other organs and the patient was not in optimal condition to undergo invasive diagnostic procedures, such as uncooperativeness, hypercapnia, hypoxemia, arrhythmia, and unstable hemodynamics.

Detection of expression of MAGE A genes

RNA was extracted from core biopsies specimens using RNAeasy Plus Mini Kit (Qiagen, Hilden, Germany) with the procedure in the protocol as in our previously study (Mastutik et al., 2021). Total RNA was used as a template for reverse-transcription PCR (RT-PCR) with the RT PCR Master Mix (Toyobo, Osaka, Japan) and followed by nested PCR.

Before RT PCR was performed to detect MAGE A expression, all samples were used for RT PCR using the housekeeping gene, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH). This aims to ensure that the specimen used in the RT PCR process has DNA in sufficient quantity. Specimens with GAPDH positive will be used to identify the expression of the MAGE A family gene. If the GAPDH RT-PCR results show negative, then the sample is excluded.

Identification of MAGE A1-10 was performed the PCR using the MF10/MR10 primers and MF10/MR12 primers (Mastutik et al., 2021), while MAGE A1-6 by using MMRP1/MMRP1 primers and MMRP3/MMRP4 primers (Park et al., 2002). The GAPDH primers, the individual of MAGE A from MAGE A1 to MAGE A10 primers, and the PCR condition were performed as in the previous studies (Park et al., 2002; Mastutik et al., 2021).

Statistical analysis

The expression of MAGE A was presented in percentage. The association between the expression of MAGE A1-10, MAGE A1-6, and individual MAGE A from MAGE A1 to MAGE A10 with histopathological

finding were analysis with Fisher's Exact Test 2 sided. The sensitivity and specificity were showed in percentage.

Results

This study was conducted on 67 core biopsy specimens from patients with clinical diagnoses of lung or mediastinal tumors. The patients consisted 45 (67.2 %) lung tumors and 22 (32.8 %) mediastinal tumors, 46 males and 21 females. The age range was 18-79 years old. Most patients were aged 51-60 years old (Table 1).

The histopathological finding from the core biopsy specimens showed 41 (61.2 %) of specimens as malignant cells and 26 (38.8 %) of specimens as non-malignant cells. The malignant cells from lung tumors were carcinoma poorly differentiated (1 patient), Small cell carcinoma (1 patient), NSCLC type adenocarcinoma (23 patients), and NSCLC type of squamous cell carcinoma (2 patients), NSCLC type of adenosquamous carcinoma (2 patients), (Figure 1). In non-malignant cells were inflammation (6 patients), and no found malignant cells (10 patients). Furthermore, the malignant cells from mediastinal tumors were malignant round cell tumor (4 patients), malignant germ cell tumor (1 patients), malignant lymphoma (1 patients), hodgkin lymphoma (2 patients), and non-hodgkin lymphoma (4 patients), while in categories non-malignant cell were thymoma (2 patients) and no found malignant cells (8 patients).

The group of MAGE A1-10 was expressed the most in the lung and mediastinal tumors. There was 47 (70.1 %) for MAGE A1-10 and followed by MAGE A1-6 at 25 (37.3 %). For individual MAGE A family genes showed MAGE A5 was expressed at 44.8 %, then followed by MAGE A8 was 29.9 %, MAGE A1 was 25.4 %, MAGE A9 was 19.4 %, MAGE A3 was 10.4 %, MAGE A2 was 6.0 %, MAGE 10 was 1.5 % (Table 2). In addition, there were no specimens expressed MAGE A4 and A6 (Table 2).

From specimens with histopathological finding of malignant cells, MAGE A1-10 gene was expressed in 33 (80.5 %) and MAGE A1-6 in 19 (46.3 %). The individual of MAGE A family, MAGE A5 was expressed in 53.6 %, MAGE A8 in 41.5 %, MAGE A1 in 29.3 %, MAGE A9 in 14.6 %, MAGE A2 and A3 in 9.8 % respectively, and MAGE A10 in 2.4 % (Table 2). This showed that the MAGE A1-10 group was most highly expressed in

malignant cells.

Furthermore, in non-malignant cells, the MAGE A gene family was expressed positively. The MAGE A1-10 group were expressed on 14 (53.9 %), MAGE A1-6 on 6 (23.1 %), and the single gen of MAGE A5 was expressed on 30.8 %, MAGE A9 on 26.9 %, MAGE A1 on 19.2 %, MAGE A3 and MAGE A8 on 11.5 %, respectively (Table 2).

The MAGE A1-10 expression was significantly associated with histopathological diagnosis ($P < 0.05$) with a probability value was 0.041. The contingency coefficient value for MAGE A1-10 was 0.273 (P -value 0.020) with the strength of association being weak (0.21–0.4) (Table 2). Analysis of the diagnostic value of MAGE A1-10 showed a sensitivity of 80.5 %, a specificity of 46.2 %, and a diagnostic accuracy of 67.2 % (Table 3).

This study found that there is a significant association between the expressions of MAGE A8 with histopathology diagnosis of malignant cells and non-malignant cells ($P < 0.05$), the P -value was 0.020. The contingency coefficient value was 0.304 (P -value 0.009) with the strength of association is being weak (0.21–0.4) (Table 2).

Table 1. Characteristics of the Patient from the Core Biopsy of Peripheral Lung Tumor

Characteristic Patients		Frequency	Percentage (%)
Age (mean + SD)	50.81 + 14.770		
Sex	Male	46	68.7
	Female	21	31.3
	Total	67	100
Clinical Diagnosis	Lung Tumor	45	67.2
	Mediastinal Tumor	22	32.8
	Total	67	100
Histopathological Diagnosis	Malignant cell	41	61.2
	Non-malignant cell	26	38.8
	Total	67	100
Lung Tumor	Malignant cell	29	64.4
	Non-malignant cell	16	35.6
	Total	45	100
Mediastinal Tumor	Malignant cell	12	54.5
	Non-malignant cell	10	45.5
	Total	22	100

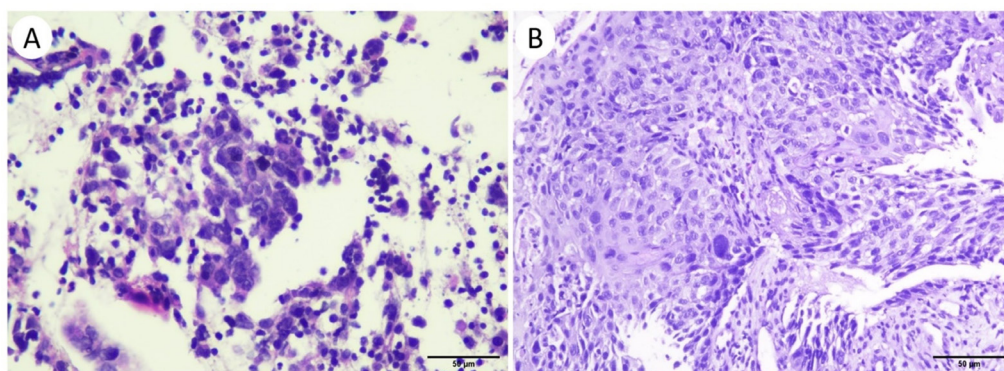


Figure 1. Histopathological Diagnosis of Lung Tumors. (A) Non-small cell lung cancer type adenocarcinoma and (B) Non-small cell lung cancer type squamous cell carcinoma observed with a light microscope, magnification 100x.

Table 2. The Expression of MAGE A Gene Family based on Histopathological Finding from the Core Biopsy of Lung and Mediastinal Tumor

Subtype of MAGE A	Histopathological finding		Total N (%)	P Value	Contingency coefficient value
	Malignant cells N (%)	Non-malignant cells N (%)			
MAGE A1-10					
Positive	33 (80.5)	14 (53.9)	47 (70.1)	0.041*	0.273 (P = 0.020)
Negative	8 (19.5)	12 (46.1)	20 (29.9)		
MAGE A1-6					
Positive	19 (46.3)	6 (23.1)	25 (37.3)	0.097	
Negative	22 (53.7)	20 (76.9)	42 (62.7)		
MAGE A1					
Positive	12 (29.3)	5 (19.2)	17 (25.4)	0.527	
Negative	29 (70.7)	21 (80.8)	50 (74.6)		
MAGE A2					
Positive	4 (9.8)	0	4 (6.0)	0.152	
Negative	37 (90.2)	26 (100)	63 (94.0)		
MAGE A3					
Positive	4 (9.8)	3 (11.5)	7 (10.4)	1,000	
Negative	37 (90.2)	23 (88.5)	60 (89.6)		
MAGE A4					
Negative	41 (100)	26 (100)	67 (100)	-	
MAGE A5					
Positive	22 (53.6)	8 (30.8)	30 (44.8)	0.113	
Negative	19 (46.3)	18 (69.2)	37 (55.2)		
MAGE A6					
Negative	41 (100)	26 (100)	67 (100)	-	
MAGE A8					
Positive	17 (41.5)	3 (11.5)	20 (29.9)	0.020*	0.304 (P = 0.009)
Negative	24 (58.5)	23 (88.5)	47 (70.1)		
MAGE A9					
Positive	6 (14.6)	7 (26.9)	13 (19.4)	0.356	
Negative	35 (85.4)	19 (73.1)	54 (80.6)		
MAGE A10					
Positive	1 (2.4)	0	1 (1.5)	1.000	
Negative	40 (97.6)	26 (100)	66 (98.5)		

The diagnostic value analysis showed that MAGE A8 had a sensitivity of 41.5 %, a specificity of 88.5 %, and a diagnostic accuracy of 59.7 % (Table 3). This study found that there is no significant association between the

Table 3. The Diagnostic Values of MAGE A Gen Family based on the Histopathological Finding of Core Biopsy Specimens.

	Sn (%)	Sp (%)	PPV (%)	NPV (%)	LR+	LR-	DA (%)
MAGE A1-10	80.50	46.2	70.2	60	1.49	0.42	67.2
MAGE A1-6	46.30	76.9	76	47.6	2.01	0.70	58.2
MAGE A1	29.23	80.8	70.6	42	1.52	0.88	49.3
MAGE A2	9.80	100	100	41.3	-	0.90	44.8
MAGE A3	9.80	88.5	57.1	38.3	0.85	1.02	40.3
MAGE A5	53.70	69.2	73.3	48.7	1.744	0.67	59.7
MAGE A8	41.50	88.5	85	48.9	3.59	0.66	59.7
MAGE A9	14.60	73.1	61.2	35.2	0.54	1.17	37.3
MAGE A10	2.40	100	100	39.4	-	0.98	40.3

Note: Sn, Sensitivity; Sp, Specificity; PPV, Positive predictive value; NPV, Negative predictive value; LR+, positive like ratio; LR-, negative like ratio; DA, Diagnostic accuracy

expressions of MAGE A1-6, MAGE A1, MAGE A2, MAGE A3, MAGE A5, MAGE A9, MAGE A10 with histopathology diagnosis of malignant cells and non-malignant cells ($P > 0.05$) (Table 2).

Discussion

Lung cancer is one of cancer that is usually diagnosed at an advanced stage. One of the obstacles encountered in diagnosing lung malignancy is the location of the deep tumor in the thoracic cavity (Marhana et al., 2021; Marhana et al., 2022). Therefore, specimens were collected in the process of diagnosing lung malignancy with interventions, such as core biopsy, fine needle aspiration biopsy, forceps biopsy, bronchoalveolar lavage, and brushing (Marhana et al., 2021). Compared with the fine needle aspiration biopsy, the core biopsy procedure showed lower complications, such as pneumothorax and hemoptysis (Yao et al., 2012). In this study, specimens from the core biopsies of peripheral lung tumors were used to establish a histopathological diagnosis and examine mRNA of MAGE A gene family. The mRNA type can be identified by nested polymerase chain reaction (PCR) (Park et al., 2002; Mastutik et al., 2007). This gene is expressed from a silent condition in normal cells or overexpressed in cancer cells before symptoms appear that may be beneficial as a biomarker in the diagnosis and prognosis of lung cancer (Jheon et al., 2004; Karimi et al., 2012; Weon and Potts, 2015).

The core biopsy specimen was a small piece of a specimen that was used in a routine procedure to determine the malignancy status of a patient. Based on the histopathological feature, it found 61.2 % of specimens as malignant cells and 38.8 % of specimens as non-malignant cells. Expression of a single MAGE A gene family showed that MAGE A5 was the most frequent, then followed by MAGE A8, MAGE A1, MAGE A9, MAGE A3, MAGE A2, and MAGE A10. It followed the previous studies in lung cancer that showed the expression of MAGE A1 was 27-46 %, MAGE A3 was 38-55 %, MAGE A4 was 19-35 %, MAGE A6 was 26 %, MAGE A10 was 14-27 % (Weon and Potts, 2015). Another study reported MAGE A3 was expressed at 42 %, MAGE A1 at 27 %, MAGE A4 at 19 %, and MAGE A10 at 14 % of lung cancer (Kim et al., 2012). MAGE A1 and MAGE A3 have the same expression that was at 77 % of SCLC and 67 % of NSCLC, MAGE A4 was expressed on 82 % of SCLC and 67 % of NSCLC (Sugita, 2002), while MAGE A3 was expressed on 73 % of NSCLC (Chen et al., 2017).

This study showed that MAGE A1-10 expression was the most frequently found, followed by MAGE A1-6 expression. It found MAGE A1-10 was expressed at 70.1 % specimens and MAGE A1-6 was expressed at 37.3 % specimens. In addition, MAGE A1-10 expression were found to be more frequently detected in malignant cells than in non-malignant cells. In malignant cells, the MAGE A1-10 expression was detected at 80.5 %, while MAGE A1-6 expression was detected at 46.3 %. In non-malignant cells, MAGE A1-10 was detected in 53.9 %, while MAGE A1-6 was detected in 23.1 %. MAGE A1-6 expression in this study was lower than previous studies, but MAGE

A1-10 expression showed almost the same number as MAGE A1-6 expression in previous studies. Previous studies have shown that MAGE A1-6 were expressed on 70 % of head and neck cancer (Noh et al., 2016). MAGE A1-6 was expressed on 71 % of oral cancer (Ries et al., 2008), on 83 % of lung cancer tissue (Jheon et al., 2004), and 15 % in bone marrow of lung cancer patients (Yi et al., 2017). It suggested that the examination of MAGE A1 to A10 and MAGE A1 to A6 expression can support each other in determining lung malignancy.

Moreover, the MAGE A gene has been reported that it was expressed in several types of cancer and may be beneficial for detecting of lung cancer in early stage and for predicting the prognosis of patients. In oral squamous cell carcinoma, the expression of the MAGE A3 to A5, and MAGE A9 were correlated to lymph node metastases and MAGE A1 was associated with clinical stage progression (Brisam et al., 2016). In advanced gastric cancer, the MAGE A1 expression was related with poor overall survival and can be served as poor prognose (Ogata et al., 2011; Lian et al., 2017). A meta-analysis study reported that the overexpression of MAGE A could be a potential marker for poor prognosis in several cancer cases (Poojary et al., 2020). Furthermore, the MAGE A gene is also expressed in the tissue surrounding NSCLC cancer which appears normal based on the histopathological diagnosis (Karimi et al., 2012) and associated with poor clinical prognosis (Weon and Potts, 2015). Expression of MAGE A gene family related to the shorter overall survival of lung cancer patients (Gu et al., 2018) and laryngeal cancer (Liu et al., 2020). It is also associated with lymph node metastasis of oral cancer and advanced-stage of disease clinically (Brisam et al., 2016).

This study found that there was a significantly different association between the expression of MAGE A1-10 and MAGE A8 with histopathological diagnosis showing malignant or non-malignant cells. In addition, 53.9 % of specimens in the group of non-malignant cells were expressed MAGE A1-10. In histopathological findings, malignant and non-malignant cells was determined based on the characteristic of structural alteration in cells or tissues. They can be observed microscopically on a slide (Gurcan et al., 2009; Jayasinghe et al., 2015; Wang et al., 2021). While examination of MAGE A expression is based on the transcription activation process to produces mRNA in the cell that may occur at the molecular level before or during the cell structure changes (Park et al., 2002). This study showed that MAGE A1-10 can still be found positive by nested PCR technique on the histopathological findings of the non-malignant cells. This could be used to improve diagnostic accuracy when cancer cells are not found in the specimen. In addition, to determine therapeutic options for lung cancer patients, it is necessary to diagnose the type of cancer based on histopathological changes. Therefore, these two examination methods can complement each other. If malignant cells are not found, then a molecular examination can be carried out using the PCR technique to find out whether the specimen contains cancer cells or not.

The gold standard in examining the malignancy status of lung tumors is histopathological examination (Gurcan

et al., 2009). Nested PCR in this study was able to detect MAGE A1 to MAGE A10 as previous study (Mastutik et al., 2021). It was compared to histopathological finding showed that MAGE A1-10 expression had a sensitivity of 80.5 % and a diagnostic accuracy of 67.2 %. MAGE A1-10 is most frequently expressed in tumor specimens with sensitivity and diagnostic accuracy of more than 65 %. Therefore, the examination of MAGE A1 to MAGE A10 expression by nested PCR technique could be used as an alternative assay method to detect cancer cells in specimens from the core biopsy of peripheral lung cancer.

The MAGE A1-10 was expressed highest in the core biopsies of peripheral lung tumors and associated with the histopathological finding of malignant or non-malignant cells. The MAGE A1-10 detection is more sensitive and specific, as well as diagnostic accuracy than the identification of MAGE A family individuals. In addition, this detection can be performed using small specimens of peripheral lung tumors taken by core biopsy. Therefore, the MAGE A1-10 assay could be beneficial to assist in the diagnosis of malignancy in lung tumors.

Author Contribution Statement

Idea, conceiving, writing the manuscript: Mastutik G. Specimen collection: Marhana IA, Rahaju AN, Mastutik G. Laboratory investigation: Amin M, Mastutik G. Histopathological data: Rahniayu A, Kurniasari N, Rahaju AN. Data collection: Trianto HF, Rahniayu A. Statistical analysis: Atika; Trianto HF. Editing the manuscript: Mastutik G; Kurniasari N. Reviewing the manuscript: Mastutik G, Rahniayu A, Marhana IA, Kurniasari N, Rahaju AN, Amin M, Trianto HF, Atika.

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Ethical Declaration

This study was approved by Ethics Committee of Dr. Soetomo Hospital, Surabaya, Indonesia with ethical clearance number 497/ Panke.KKE/ VIII/2017. Subjects participating in this study signed informed consent.

Conflict of Interest

All authors have no potential conflict of interest to disclose.

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